MEIOTIC CHROMOSOMES AND THEIR TAXONOMIC VALUE IN AMPHIBIA ANURA *

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The study of the meiotic chromosomes of the amphibia *Anura* offers various findings that we consider to be useful from a taxonomic point of view.

In the case of the male line, the techniques for the study of the meiotic chromosomes are relatively simple. Since meiosis takes place entirely in the testicle, the chromosomes may be studied on slices of that organ, prepared with the normal histological techniques (cf. 5; 6), or on squashes of fragments of the testicle itself. With this latter method, which is more used today on account of its simplicity and the better results that normally ensue from it for these studies, good non-permanent preparations may be obtained in a few minutes by covering fragments of the testicle, immediately after its removal, with acetic orcein or acetic carmine (for 10-20 minutes) and crushing them between two glass slides. In order to obtain permanent preparations, the testicle is fixed for one hour in a mixture of ethyl alcohol (3 parts) and acetic acid (1 part); it is then softened in a 45% acetic acid/water solution for a few hours and the small fragments of it are crushed between two slides; these are then placed in 45% acetic acid to cause them to detach and may be stained separately with some nuclear stain, dehydrated, cleared and mounted by the normal histological techniques [the manual by Darlington and La Cour (3) may be consulted on the subject].

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The techniques for the study of the meiotic chromosomes in the female line are more complex, owing to various factors, one of the more important being the considerable dimensions reached by the oocyte and the particular ways in which oogenesis takes place, not all of it occurring in the ovary (cf. 21). Until nearly the end of the so-called first period of growth, when the oocyte nucleus is still relatively small, this study may be carried out by the technique of slices (cf. 6); shortly after the beginning of the second period of growth the diplotene bivalents become unspiralised and are enriched with numerous lateral filaments (mostly known by the name of loops), assuming the typical aspect of lampbrush chromosomes. The bivalents retain this form until just before ovulation, when they go into diakynesis, spiralising and resuming a morphology comparable to that of the bivalents of the male, and then pass rapidly into metaphase I (2, 9). The study of the diakynetic or metaphase bivalents of the oocyte may be carried out on histological sections or, more profitably, on squashes of portions of the animal pole of the oocyte (19).

On the other hand, the study of the diplotene bivalents, in the form of lampbrush chromosomes, requires entirely particular techniques, since these chromosomes are too long to be analysed conveniently on sections (in the *Anura* they are of the order of 100 $\mu\mu$ or more) and are too transient for crushing. The most suitable techniques for the study of the lampbrush chromosomes, introduced by DURYEE (4), were subsequently perfected chiefly by GALL (8) and by CALLAN *et al.* (1); for details, we therefore refer to the works of these authors.

Our researches on the diplotene bivalents of the female of the *Anura* are essentially intended to identify any differences in the frequency and localisation of the chiasmata between the chromosomes of different species; for this reason we have not generally taken account of the form and structure of the chromosome loops; in fact, we have tried to obtain bivalents devoid of loops, so that they might more clearly reveal the disposition of the chiasmata on the homologues. The techniques that we used are briefly described below (after GALL, 1964).

The animal (an adult female) is anaesthetized with MS 222 (Sandoz) (0.1% solution in tap water); with a lateral incision in the abdomen the peritoneal cavity is opened and a fragment of ovary is removed; the wound may be closed with catgut and the animal can recover and serve for the removal of further fragments. The fragment of ovary is placed in a dry embryological watch-glass and, if the latter is sealed with a flat glass cover, may be used for several days as a source of oocytes, by keeping it in a refrigerator at + 4° C. The oocytes that are to be studied immediately (of about 1 mm. in diameter) are placed in a physiological solution free from Ca, for the composition of which we refer to the work of CALLAN and LLOYD.

The oocyte is carefully opened towards the animal pole with jewellers' tweezers, in such a way as to allow the nucleus and part of the yolk to emerge; the nucleus is then cleaned of the yolk with needles and transferred with a pipette into a flat-bottomed well-slide made out of a glass slide in which a hole has been drilled and closed on the lower side with a coverslip attached with paraffin. In this well-slide, filled with a buffered physiological solution, the nucleus is delicately deprived of the membrane, using very fine needles; in this way the nuclear sap and the chromosomes spread rapidly over the bottom of the well-slide; the latter may be closed at the top with a second coverslip and the lampbrush chromosomes may be preserved for some days in this solution, if they are kept in a refrigerator. We have used solutions that are more hypotonic than those used by the authors quoted (4, 8, 1), in order to obtain, in a short time, the detachment or retraction of the normal chromosome loops and, presumably, of a large part of their fusions, which may simulate chiasmata. The chromosomes, spread over the bottom of the well-slide, may be analysed with an inverted microscope, of the type use for *in vitro* cultures, by using phase-contrast lenses (we used a Zeiss plankton microscope with a Ukatron 60 camera attached). At a low degree of magnification, and if stained with some vital stain (toluidine blue, much diluted, in the solutions in which the nucleus is opened), the lampbrush chromosomes may be observed and photographed with a normal microscope (Figs. 1 - 2 have been reconstructed by using photograms taken with the C. Zeiss Standard Photomicroscope). In some cases the lampbrush chromosomes can be prepared in a permanent manner by prefixing them in the well-slide (open) with formalin vapour, detaching from the glass slide the coverslip to which they are attached and fixing and staining the chromosomes directly on the slide.

Let us now consider some of the results that we obtained by using these techniques and their value in the problems involved in the systematic classification of the *Anura*.

As regards the diplotene of the female, our observations are concerned with four species of *Anura;* one of them belongs to a primitive familiy *(Bombina variegata:* Discoglossidae) and three to families considered evolved *(Bufo viridis:* Bufonidae; *Hyla arborea: Hylidae; Rana esculenta:* Ranidae); some of these observations have already been published (11,7). In all these species, the diplotene bivalents, devoid of most of the loops, present a morphology that differs only slightly in the various species: the chiasmata, which are generally more than two in number in the large bivalents and two or less in the small ones, have no precise localisation, being arranged randomly along the chromosome axes; the terminal chiasmata are less frequent. There are differences in the average number of chiasmata per chromosome in the various species, though they are fairly small and cannot be assessed with certainty, owing to the doubt that exists as to whether or not possible fusions of homologous loops (which simulate chiasmata) survive the hypotonic treatment. Genetically, the most interesting fact that emerges from our observations therefore seems to us to be that of the unrestricted distribution in the chiasmata on the bivalents of the females of various families of *Anura*, which seems likely to represent a general condition, since the observations were made on specimens of all four families studied. Not having studied the stages following the diplotene, we can say nothing about possible differences between the various species in the phenomena of the terminalisation of the chiasmata and of the chromosome spiralisation, which are established in the diakinesis and in metaphase I.

As regards the male line, we have studied the meiosis of numerous species of nearly all the families of *Anura* (Ascaphidae, Discoglossidae, Pipidae, Pelobatidae, Microhylidae, Leptodactylidae, Hylidae, Bufonidae, Ranidae and Hyperolidae; cf. 5, 11, 12, 13, 14, 15).

In the stages that go from the diplotene to metaphase I, the *Anura* studied reveal considerable morphological differences, which allow them to be subdivided into two groups of families, with some uncertainty as regards the Pipidae.

In *Ascaphus truei* (Ascaphidae), *Discoglossus pictus, Bombina variegata, B. bombina, Alytes obstetricans* (Discoglossidae), the spermatocyte diplotene is a very long stage, during which the bivalents, relatively little spiralised, have very clear outlines and reveal chiasmata, mostly interstitial, without any precise localisation. There are generally more than two chiasmata in the large bivalents and only one in the small bivalents. On the other hand, the diakinesis is a fairly short stage, during which the chromosome spiralisation and the number and terminalisation of the chiasmata undergo relatively small changes compared with those undergone by the diakinetic chromosomes of the other *Anura*. At metaphase I the large bivalents show 2 or 3 chiasmata, the small bivalents generally only one; only some of these chiasmata terminalise; the chromosome spiralisation often does not reach the high levels attained by the metaphase bivalents of the other *Anura* (Figs. 3, 5, 7, 9).

These conditions are revealed with some differences in the 4 genera studied and there are sometimes variations between the figures of the same individual, though these appear infrequent; in general, *Bombina* and *Ascaphus* follow the pattern closely, while *Alytes* and *Discoglossus* reveal more highly spiralised bivalents and the less frequent presence of chiasmata compared with the first two genera.

In the other families of *Anura*, the diplotene of the male line is a very short stage, at the beginning of which the bivalents have an ill-defined morphology, appearing relatively spiralised but entangled and with unsharp and spiny outlines. About the middle and late diplotene stage, when the homologues, continuing to spiralise and diverging, assume a more sharply definable morphology, the chiasmata appear already terminal in almost every case; their number which cannot always be well defined before the late diplotene stage, is about 2 for every bivalent (cf. 5, 12, 15). The diakinesis is very long; at this stage the bivalents are seen to be intensely spiralised and all provided with two terminal chiasmata each, which give them a typical ring shape. This morphology is retained until metaphase I, when a further increase in the spiralisation occurs (Figs. 4, 6, 8, 10).

The essential morphological characteristics of the male meiosis of the two groups of families of *Anura* are summarised in the Table.

TABLE

Characteristics of the meiosis of the ♂	Ascaphidae and Discoglossidae	Other families of *Anura*
Relative length of stages of late prophase I	Long diplotene Short diakinesis	Short diplotene Long diakinesis
Degree of spiralisation of the bivalents in late prophase I	Bivalents relatively little spiralised	Bivalents highly spiralised
Average number of the chiasmata in late diplotene and in diakinesis	More than two chiasmata in the large bivalents; one only in the small bivalents	Two chiasmata in both large and small bivalents *, **
Position of the chiasmata in late diplotene and in diakinesis	Interstitial chiasmata in the majority	Terminal chiasmata *, **
Terminalisation of the chiasmata at metaphase I	Normally never total	Normally total *

* Except for *Pipa parva*, with bivalents mostly provided with a single procentric chiasma.

** Some African Ranidae and Hyperolidae have a certain number of meiotic figures with bivalents having three chiasmata.

In the Pipidae, the male meiosis is of the ring-shaped bivalent type in the two African genera *Xenopus* and *Hymenochirus*, while in the

American *Pipa parva* it is of an entirely particular type, which may perhaps be associated with that of Ascaphidae and Discoglossidae: in this species, the meiotic bivalents often reveal a single chiasma, mostly procentrically localized (13) (Fig. 11).

Among the higher *Anura*, a partial exception to the pattern is represented by certain African Ranidae *(Pyxicephalus)* and certain Hyperolidae *(Kassina, Hyperolius)*, in which some of the meiotic figures of the late diplotene and the diakinesis present bivalents that are sometimes provided with interstitial chiasmata (Fig. 12).

The data so far obtained on the morphology of the meiosis of the male in the *Anura* offer us a clearly dichotomous division among them: on the one hand, Ascaphidae and Discoglossidae; on the other hand, the remaining families so far studied, with the partial exceptions now described. It is to be noted that the differences in the male meiosis among the *Anura* do not seem to be explainable on the basis of differences in the chromosome characteristics, because the morphology, dimensions and number of the mitotic chromosomes appear very similar, in many cases, between species of *Anura* that have a different type of male meiosis.

The meiosis of the male of Ascaphidae and Discoglossidae shows clear similarities with the meiosis of the male of many *Urodela* and of the few *Gymnophiona studied* (5, 18, 10); these data, on the one hand, favour the hypothesis of a karyological monophyletism of the present *Amphibia* (cf. 20); on the other hand, they prove the karyological primitiveness of Ascaphidae and Discoglossidae, which, as regards the characteristics of the meiosis, are more similar to the *Urodela* (probably older than the *Anura)* and the *Gymnophiona*, than to the higher *Anura*. However, even if the type of spermocytarian meiosis with ring-like bivalents may be considered to be derived, it must have appeared very early in the phylogenesis of the *Anura*, since it is also present in the Pipidae, which are one of the oldest families in the Order (17).

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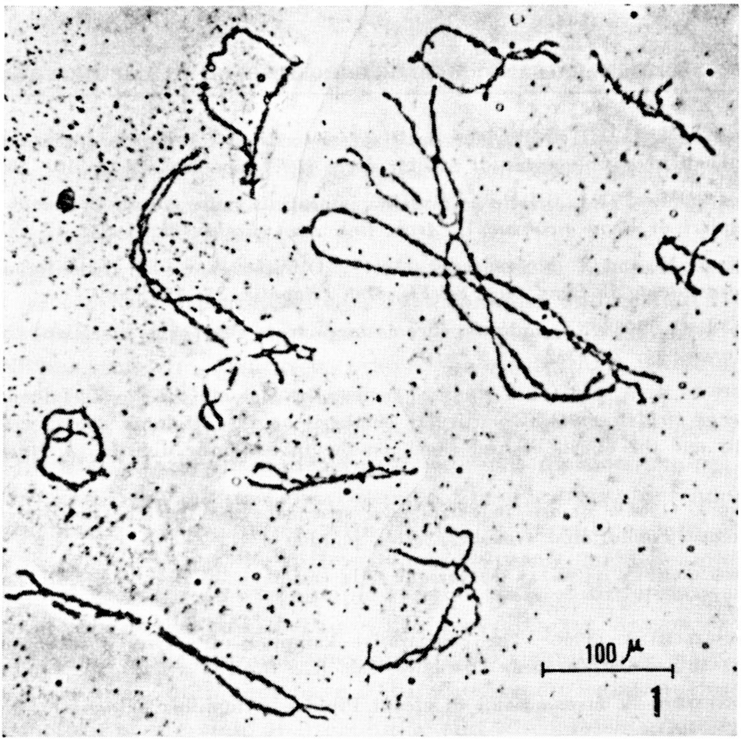

FIGURE 1. Oocyte bivalents of *Rana esculenta*. This figure was reconstructed using many photograms, given the large dimensions of the chromosomes.

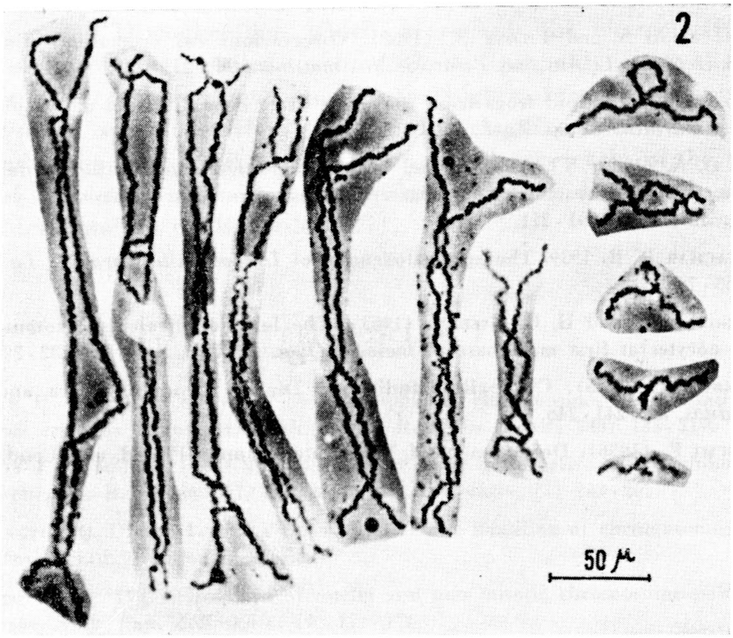

FIGURE 2. Oocyte bivalents of *Bombina variegata*, ordered in decreasing length.

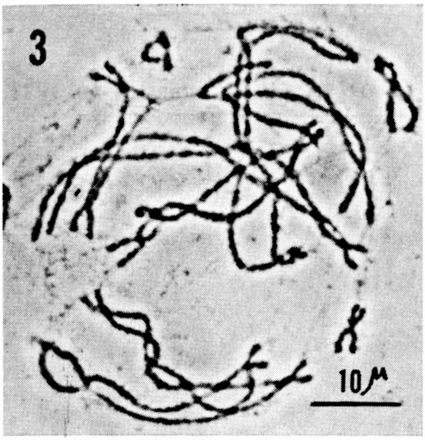

FIGURE 3. Early spermatocyte diplotene of *B. variegata.*

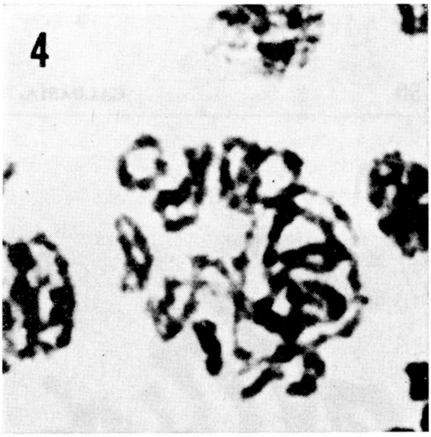

FIGURE 4. (Figs. 4-8 are at the same enlargement, the same as in Fig. 8): Early spermatocyte diplotene of *R. esculenta.*

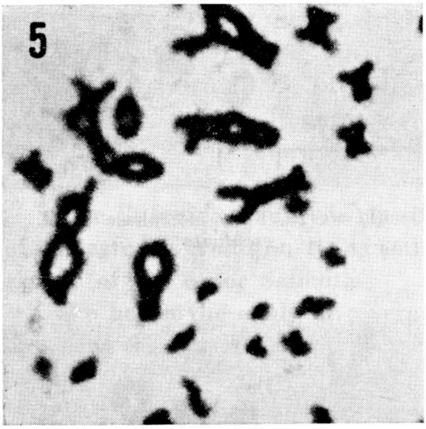

FIGURE 5. Late spermatocyte diplotene of *Ascaphus truei.*

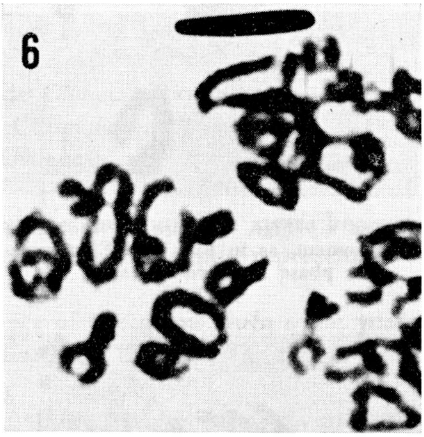

FIGURE 6. Late spermatocyte diplotene of *R. esculenta.*

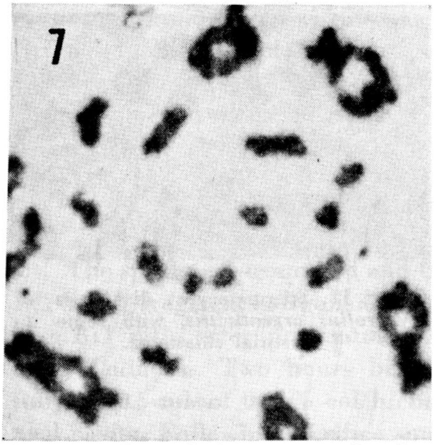

FIGURE 7. Late diplotene or early diakinesis of *Alytes obstetricans* ♂.

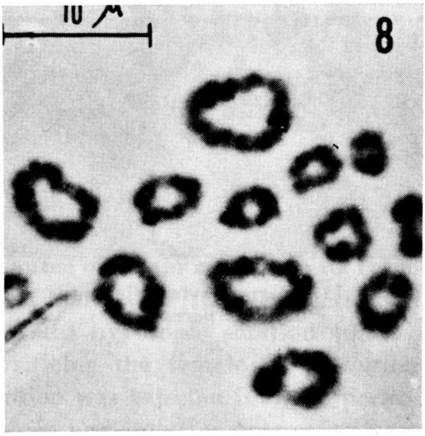

FIGURE 8. Early diakinesis of *Kassina wealii* ♂ (Hyperolidae).

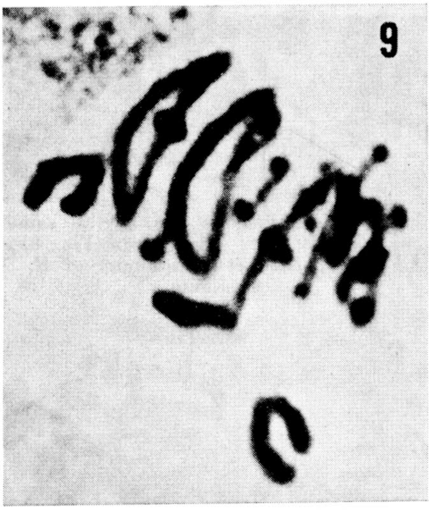

FIGURE 9. (Figs. 9-12 are at the same enlargement, as in Fig. 10): First metaphase in *B. variegata* ♂.

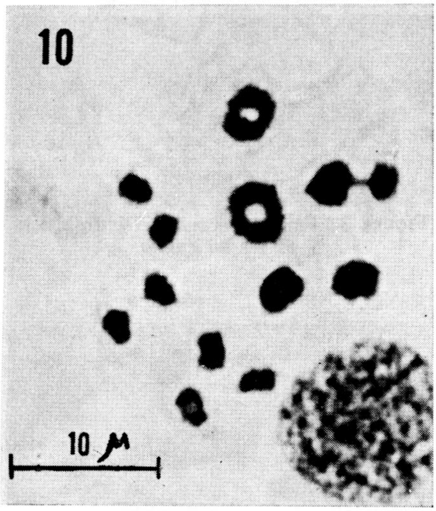

FIGURE 10. First metaphase of *R. esculenta* ♂.

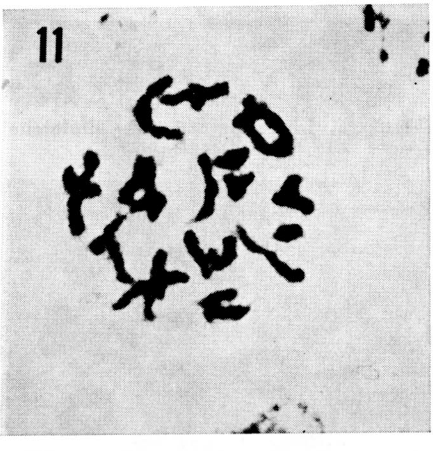

FIGURE 11. Spermatocyte diakinesis of *Pipa parva*.

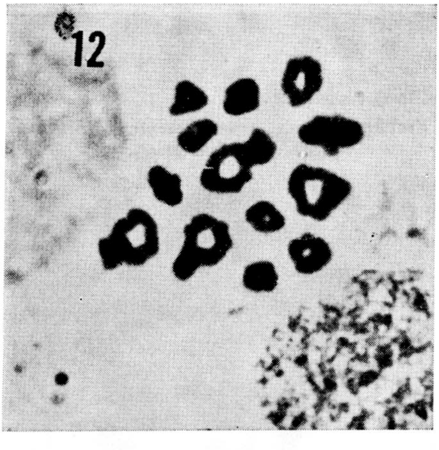

FIGURE 12. Spermatocyte diakinesis of *Hyperolius argentivittis*, with some interstitial chiasmata.